Chapter 3
Second Section: A single domain antibody detects and neutralises toxic $A\beta_{42}$ oligomers in the Alzheimer's disease CSF

1. DesAb-O selectively detects synthetic $A\beta_{42}$ oligomers *in vitro*

We firstly evaluated the specificity of DesAb-O against different $A\beta_{42}$ conformers by dot- blot analysis. $A\beta_{42}$ conformers were assembled *in vitro* according to well-defined protocols. Thus, toxic A+ and nontoxic A- oligomers (Ladiwala *et al.* 2012), toxic $A\beta_{42}$ ADDLs (Lambert *et al*. 1998) and two types of fibrils, namely F1 (Ladiwala *et al.* 2012) and F2 (Dahlgren *et al*. 2002) were formed and their identities were confirmed by routinely analysing them by atomic force microscopy (AFM) and dot-blots using their respective conformation-sensitive Abs, as previously reported (Banchelli *et al.* 2020). The various $A\beta_{42}$ species were then deposited (2 µl, corresponding to 0.1 µg) onto a nitrocellulose membrane and detected with the different Abs. As controls, we used the 19.3 Ab, specific for amyloid β-derived diffusible ligands (ADDLs) (Savage *et al*. 2014), A11 Ab, recognizing toxic oligomers from various proteins, but not their monomeric or fibrillar conformations (Kayed *et al.* 2003), and OC Ab, specifically raised against fibrillar aggregates (Kayed *et al.* 2007).

DesAb-O was found to selectively target toxic A+ oligomers and ADDLs, with a minor cross-reaction with nontoxic A- oligomers (Figure 3.1 A). As expected, each of the above mentioned control Abs bound to their targeted conformers according to previous reports (Kayed *et al.* 2003; Kayed *et al.* 2007; Ladiwala *et al.* 2012; Savage *et al*. 2014; Bigi *et a.*, 2020). Proper loading of each $A\beta_{42}$ species was confirmed by the sequence-specific 6E10 Ab which targets the N-terminus of $A\beta_{42}$ (Kim *et al*. 1990; Kayed *et al*. 2003; Kayed *et al*. 2007;

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Book References DOI 10.36253/979-12-215-0993-9.references

Baghallab *et al*. 2018), which bound all the analysed $A\beta_{42}$ species, independently of their aggregation state (Figure 3.1 A).

We then assessed the sensitivity of DesAb-O by evaluating its ability to probe decreasing amounts of $A\beta_{42}$ conformers (0.10, 0.05, 0.025, 0.01 and 0.005 μg), previously deposited onto a nitrocellulose membrane (Figure 3.1 B). DesAb-O was found to detect A+ oligomers and ADDLs down to 0.01 μg, which when compared to the control 19.3 Ab, appeared more sensitive (Figure 3.1 B).

The reactivity of DesAb-O against $A\beta_{42}$ species was also quantified by performing an indirect ELISA assay. Briefly, we coated the wells of the ELISA plates with increasing concentrations of $A\beta_{42}$ conformers (0, 1, 5 and 10 μM). We then incubated the plates with DesAb-O and subsequently with the appropriate secondary Abs. Our results showed that DesAb-O clearly recognized toxic A+ oligomers and ADDLs at 5 and 10 μM, with a specificity that increased with aggregate concentration (Figure 3.1 C), revealing no affinity for the monomeric or fibrillar forms even at high concentrations (Figure 3.1 C). The 19.3 Ab was also assessed for a relative comparison, revealing high affinity for A+ oligomers and ADDLs, even at 1 μM for the former species, but showing a minor specificity for $A\beta_{42}$ monomers (Figure 3.1 D). As expected, the 6E10 Ab, was found to detect all the analysed $A\beta_{42}$ conformers in a dose-dependent manner (Figure 3.2), as the $A\beta_{42}$ N-terminus is solvent-exposed regardless of the aggregated state of the peptide (De *et al*. 2019). Overall, dot-blot and ELISA results demonstrate the ability of DesAb-O to selectively discriminate $A\beta_{42}$ conformers, in agreement with previously established work (Aprile *et al*. 2017; Aprile *et a*. 2020). Moreover, the affinity of DesAb- O against $A\beta_{42}$ oligomers and its selectivity for the oligomeric species with respect to the monomers and fibrils do not seem to be any worse than those observed with commercially available Abs, providing the platform for further applications.

2. DesAb-O detects synthetic $A\beta_{42}$ oligomers bound to neuronal membrane and internalized into the cytosol

To analyse whether or not the ability of DesAb-O to selectively detect $A\beta_{42}$ oligomers *in vitro* are also reflected in cultured cells, human neuroblastoma SH-SY5Y cells were exposed for 1 h to different types of $A\beta_{42}$ species at 3.0 μM (monomer equivalents) and then the plasma membrane (red channel) and $A\beta_{42}$ aggregates (green channel) were counterstained and analysed by the super-resolution stimulated emission depletion (STED) microscope (Cascella *et al.* 2021) (Figure 3.3 and 3.4). DesAb-O was found to detect toxic A+ oligomers and ADDLs (Figure 3.4 A), showing an increase of the green fluorescent signal by 1362 ± 46% and 1010 ± 45%, respectively, with respect to the untreated cells, taken as 100% (Figure 3.4 B). In particular, by analysing different optical sections (apical, median and basal planes to the coverslip) DesAb-O can identify these oligomers bound to the neuronal membranes and penetrating into the cells

(Figure 3.3).

Similar results were obtained with the 19.3 Ab (1010 ± 99% and 1235 ± 39%) and to a lower extent with the A11 one (680 ± 66% and 633 ± 97%) (Figure 3.4 A,B). Interestingly, the 19.3 and A11 antibodies exhibited a green fluorescence signal in foci, in contrast to the diffused green fluorescence observed in DesAb-O. Indeed, this variation can be attributed to the small dimensions and the high sensitivity of the sdAb for oligomeric conformers, which facilitates binding to a higher number of epitopes on the oligomer surface. OC Ab specifically recognized both types of fibrillar conformers (F1 and F2), which appeared predominantly bound to the plasma membranes, and the green fluorescent signal increased by 557 ± 55% and 562 ± 57% for the former type of fibril (Ladiwala *et al*. 2012) and the latter type of fibril (Dahlgren *et al.* 2002), respectively (Figure 3.4 A,B). As expected, the sequence-specific 6E10 Ab detected both A+ oligomers and ADDLs and F1 and F2 fibrils on neuronal cells (Figure 3.4 A,B). None of the Abs detected nontoxic A- oligomers, that evoked a very low and undetectable fluorescent signal because they are known to weakly interact with neuronal membranes (Ladiwala *et al.* 2012; Banchelli *et al*. 2020; Bigi *et al*. 2020).

Overall, in our experimental conditions DesAb-O selectively discriminated toxic $A\beta_{42}$ oligomers with respect to monomeric and fibrillar forms of the peptide, at least similarly to the other commercially available conformation-sensitive Abs, suggesting a very promising potential for the detection of harmful $A\beta_{42}$ species in biological fluids.

3. DesAb-O inhibits the interaction of $A\beta_{42}$ oligomers with neuronal membranes preventing mitochondrial dysfunction

We further evaluated whether DesAb-O was also able to capture $A\beta_{42}$ oligomers, preventing their detrimental effects on neuronal cells. To this purpose, A+ oligomers and ADDLs were pre-incubated for 1 h with DesAb-O or the A11 Abs at increasing molar ratios between oligomers and Abs (from 1:0.1 to 1:1), and these solutions were then added to the cell culture medium of SH-SY5Y cells for 15 min. Unlike previous experiments, the oligomers were added to cultured cells only after pre-incubation with DesAb-O. To detect only the oligomers bound to the cell surface, the cellular membrane was not permeabilized at this stage, thus preventing antibody internalization. The binding affinity of the aggregates for cellular membranes was assessed by confocal microscopy using the 6E10 Ab as a probe. Our results showed a strong colocalization of $A\beta_{42}$ A+ oligomers and ADDLs with the neuronal membranes in the absence of the pre-incubation with Abs (Figure 3.5 A), confirming previously reported data (Schengrund 2010; Evangelisti *et al.* 2013; Bigi *et al*. 2020). Notably, the binding of both types of $A\beta_{42}$ aggregates was significantly reduced in the presence of DesAb-O up to 1:0.1 molar ratio (by 40 ± 3% and 36 ± 2%, respectively) (Figure 3.5 A,C). The same analysis was performed with the A11 Ab, which was found to prevent the

interaction of the oligomers with the membrane only at 1:1 molar ratio (by 44 ± 5% and 48 ± 7%, respectively) (Figure 3.5 B,D). These results again suggested a great affinity of DesAb-O for the oligomers, at least equal to that of a traditional conformation-sensitive Ab.

We then evaluated whether DesAb-O was also able to prevent the neurotoxic effects evoked by $A\beta_{42}$ aggregates, by analysing their mitochondrial status with the MTT reduction test. $A\beta_{42}$ species (3.0 μM) were incubated in the absence or presence of an equimolar concentration of DesAb-O for 1 h, and then these solutions were added to the culture medium of SH-SY5Y cells for 24 h. Our results showed that A+ oligomers and ADDLs significantly reduced (by 31 ± 3% and 35 ± 3%, respectively) the mitochondrial activity of SH-SY5Y cells as compared to untreated cells (Figure 3.5 E), as previously shown (Kayed *et al*. 2003; Ladiwala *et al*. 2012; Evangelisti *et al*. 2016; Cascella *et al*. 2017; Bigi *et al.* 2020). Both types of fibrils were also found to be significantly toxic, even if to a lesser extent with respect to the oligomeric species (the reduction of cell viability was 21 ± 2% and 17 ± 2% for F1 and F2, respectively, as reported in Figure 3.5 E), confirming previous data (Dahlgren *et al*. 2002; Ladiwala *et al*. 2012; Bigi *et al*. 2020). When A+ oligomers and ADDLs were pre-incubated for 1 h in the presence of DesAb-O, we observed a significant improvement of mitochondrial function (by 18 ± 4% and 11 ± 3%, respectively, with respect to cells treated with the same species in the absence of DesAb-O), whereas the fibrils-induced neurotoxicity was not affected by DesAb-O (Figure 3.5 E), confirming again its high specificity in the targeting of $A\beta_{42}$ oligomeric conformations. The same analysis was performed with the 19.3 and A11 Abs, that were found to significantly prevent the cytotoxicity induced by A+ oligomers (increase of MTT reduction by 15 ± 5% and 18 ± 4%, respectively) and ADDLs (by 13 ± 3% and 22 ± 3%, respectively), whereas the OC Ab markedly suppressed the cytotoxicity of fibrillar conformations (by 16 ± 4% and 16 ± 5%, respectively), as already shown (Bigi *et al*. 2020). Of note, DesAb-O did not affect neuronal viability when added alone to the cell culture medium, thus making it an excellent tool for future tentative therapeutic applications.

4. DesAb-O detects $A\beta_{42}$ oligomers in the CSF of AD patients *in vitro*

Considering the encouraging data obtained with DesAb-O both *in vitro* and in cultured cells, we then assessed its ability to identify $A\beta_{42}$ species that are present in the CSF of AD patients with respect to the CSF of age-matched control subjects. We performed a series of proof-of-concept experiments on a small set of clinical samples of CSF (n = 9 from AD and n = 4 from controls) to explore whether our assays could detect differences between the two groups. We first performed a sandwich dot-blot analysis by spotting 2 μl of the capture Abs 6E10 and DesAb-O (corresponding to 0.01 mg/ml and 10 μM, respectively) onto a nitrocellulose membrane that was then incubated with 1.5 ml of $A\beta_{42}$ species at 2 μM or CSF from AD patients and controls at 0.1 mg/ml. The membranes were

then revealed with the 6E10 Ab. This approach, which is different from the classical dot-blot employed in Figure 3.1 A,B, is useful in a context in which the amount of oligomers we expected to have in the CSF of AD patients was very low. The 6E10 Ab, as expected, showed positive signal with all samples, including the CSFs (Figure 3.6 A). Despite the improved sensitivity in the recognition of oligomeric species, that gave rise to a high signal (Figure 3.6 A), DesAb-O was also found to generate a slight cross- reaction with monomers and to a minor extent with fibrils. Interestingly, an intense spot was observed following the incubation of DesAb-O with AD CSF and only a weak signal with the control CSF (Figure 3.6 A).

To further demonstrate the capability of DesAb-O to identify $A\beta_{42}$ oligomers in the CSF of AD patients with respect to control individuals, we performed a sandwich indirect ELISA assay, again to improve specificity and sensitivity. Briefly, we coated the ELISA plate with 1 μM DesAb-O and incubated with decreasing concentrations (4500, 2250, 450, 45, 4,5 and 2,25 pg/ml) of $A\beta_{42}$ species ($A\beta_{42}$ monomer (M), A+ oligomers, and fibrils (F1) prepared according to Ladiwala *et al*. 2012), the CSF from AD patients (n = 9) and that from control subjects (n = 4) at the concentration of 0.25 mg/ml. The plates were then probed with 6E10 as detection Ab. We found that DesAb-O significantly recognized A+ oligomers (Figure 3.6 B, orange bars) down to 2.25 pg/ml with respect to the monomeric and fibrillar forms of $A\beta_{42}$ (Figure 3.6 B, blue and grey bars, respectively), showing a lower affinity for these forms only at the highest concentration. As a control, we used monomeric αSyn, which was not recognized by DesAb-O, demonstrating again its high specificity for $A\beta_{42}$ (Figure 3.6 B, yellow bar). Notably, DesAb-O generated a high signal with the CSFs of AD patients (Figure 3.6 B, magenta bar) that appeared significantly different from that obtained with those of control subjects (Figure 3.6 B, green bar).

The ability of DesAb-O to detect $A\beta_{42}$ oligomers in the CSFs of AD patients was also exploited by super-resolution STED microscopy (Cascella *et al*. 2021). $A\beta_{42}$ species (25 μM) and CSFs from AD patients and controls (0.5 mg/ml) were deposited on a glass coverslip and labelled with 6E10 and DesAb-O Abs. As expected, the 6E10 Ab clearly recognized both preformed A+ oligomers and fibrils, even in a 1:1 mixture between oligomeric and fibrillar species (Figure 3.6 C). In particular, A+ oligomers exhibited green-fluorescent punctae, which appeared to be small and globular at the very high magnifications allowed by STED microscopy, whereas F1 appeared fibrillar in morphology (Figure 3.6 C, bottom box magnifications). Monomeric $A\beta_{42}$ is difficult to detect (Figure 3.6 C), in agreement with the results obtained in a cellular context (Figure 3.6 A,B). For this reason, the 6E10 Ab did not detect Aβ species in control CSF AD samples, whereas it did with AD CSFs, recognizing green fluorescent punctae, globular in shape, some of which apparently larger than oligomeric species (Figure 3.6 C, bottom box magnifications). In contrast, DesAb-O clearly recognizes A+ oligomers rather than fibrils, revealing the presence of small globular green fluorescent punctae in the solutions containing 1:1 mixture (Figure 3.6 C, bottom box magnifications). We then evaluated the CSFs, observing that DesAb-O can

selectively detect small, globular and round species compatible with $A\beta_{42}$ oligomers in the CSFs of AD patients, displaying no signal in the control ones (Figure 3.6 C, bottom box magnifications).

To validate our experimental approach, we tested another sdAb named $DesAb_{18–24,}$ which has been rationally designed to target $A\beta_{42}$ fibrils, specifically the region VFFAEDVG (De *et al*. 2019; Aprile *et al*. 2017). We thus performed a sandwich indirect ELISA assay by coating the wells with 0.5 μM $DesAb_{18–24}$ and then performing the test as described above. Our results showed that $DesAb_{18–24}$ clearly recognizes $A\beta_{42}$ fibrils down to 4.5 pg/ml, with low affinity for monomeric $A\beta_{42}$ down to 4500 pg/ml and no binding for A+ oligomers, except at the highest concentration (Figure 3.7 A). Notably, $DesAb_{18–24}$ was found to generate a higher absorbance value from the control CSFs with respect to that observed from AD patients, because of the high cross-reaction with the monomers (Figure 3.7 A), confirming the difference in terms of total $A\beta_{42}$ between patients and controls reported in literature.

The specificity of $DesAb_{18–24}$ was also evaluated by STED microscopy, showing a clear detection of preformed $A\beta_{42}$ fibrils, without any signal for both CSFs as the monomeric protein is difficult to reveal in this experimental condition (Figure 3.7 B).

5. DesAb-O detects $A\beta_{42}$ oligomers present in AD CSFs upon their interaction with neuronal cells

To further evaluate the ability of DesAb-O to target $A\beta_{42}$ oligomers present in the CSFs of AD patients, we applied high resolution STED microscopy to SH-SY5Y cells exposed to ADDLs, or to the CSFs of AD patients and age-matched control subjects (without pre- incubation with Abs). Following the administration for 5 h of ADDLs at 3.0 μM (monomer equivalents) or CSFs diluted 1:1 with the extracellular medium, the $A\beta_{42}$ aggregates (green channel) were counterstained with DesAb-O or 6E10 Abs and cell membrane (red channel) with wheat germ agglutinin (WGA, Figure 3.8 A,B). Cells exposed to ADDLs exhibited green-fluorescent punctae, which appeared to be small and globular at the very high magnifications allowed by STED microscopy (Figure 3.8 A,B, bottom image magnification). The DesAb-O derived green-fluorescent signals were consistent with the results obtained with the 6E10 Ab. In particular, the semi-quantitative analysis revealed that the oligomeric species are localised both intracellularly and extracellularly. Notably, DesAb-O can recognize a number of small and globular intracellular and extracellular $A\beta_{42}$ species in the CSFs of AD patients, with a morphology that resembles that of the oligomeric species (Figure 3.8 A, bottom image magnification). In addition to the small oligomeric species, the 6E10 Ab can also recognize larger aggregates in the CSFs of AD patients that, at high magnification, appeared round in morphology (Figure 3.8 B, bottom image magnification). In contrast, cells treated with the CSFs of control subjects and counterstained with both DesAb-O and 6E10 showed the presence of few $A\beta_{42}$

aggregates outside the cells or attached to the membrane, which probably represent nontoxic oligomers that are not able to permeabilize the cell membrane or low amounts of toxic oligomers that do not manifest their toxicity due to their small quantity (Figure 3.8 A,B, bottom image magnification).

Similar results were obtained in primary rat cortical neurons exposed to AD and control CSFs and labelled with DesAb-O (Figure 3.8 C).

6. DesAb-O prevents neuronal dysfunction induced by the CSFs of AD patients.

We finally evaluated whether DesAb-O can also neutralise the cytotoxicity of oligomers present in the CSFs of AD patients. A relatively high volume (ml of sample) is required to perform these experiments, so they were performed on 4 AD and 4 age-matched control CSFs. We first monitored the dysregulation of cytosolic Ca^{2+} homeostasis, which is an early upstream event evoked by extracellular $A\beta_{42}$ oligomers both in cultured neuronal cells and in relevant mouse AD models, where Ca^{2+} ions flow from the extracellular space to the cytosol (Demuro *et al*. 2005; Arbel-Ornath *et al*. 2017; Cascella *et al*. 2021; Fani *et al*. 2022; Bigi *et al*. 2023a; Bigi *et al*. 2023b). SH-SY5Y cells were treated for 5 h with the CSFs from AD patients and control subjects diluted 1:1 with the cell culture medium, following or not a 1 h pre-incubation with DesAb-O at 3 μM. The CSFs of AD patients caused a significant influx of Ca^{2+} ions (by 230 ± 11%) relative to untreated cells (Figure 3.9 A), whereas the CSFs of control subjects generated only a slight and non- significant alteration of Ca^{2+} homeostasis (Figure 3.9 A). A 1 h pre-incubation with DesAb-O significantly reduced the effect induced by the CSFs of AD patients (by 77 ± 20%), without affecting that observed in cells treated with the control ones (Figure 3.9 A). As a positive control, ADDLs were found to generate an extensive Ca^{2+} influx (by 405 ± 15%, Figure 3.9 A), that markedly decreased (by 250 ± 26%) following a 1 h pre-incubation with DesAb-O. Similar results were obtained in primary rat cortical neurons exposed for 2.5 h to the CSFs of AD patients and control subjects following or not a 1 h pre-incubation with DesAb-O at 3 μM (Figure 3.9 B). These results confirm the high specificity of DesAb-O in the targeting of neurotoxic $A\beta_{42}$ conformers present in AD CSFs.

The protective effect of DesAb-O was also observed from the analysis of the alteration of membrane permeability induced by $A\beta_{42}$ oligomers, monitoring the release of the fluorescent probe calcein-acetoxymethyl (AM), previously loaded into the cells (Cascella *et al*. 2017). SH-SY5Y cells, pre-loaded with calcein-AM, were treated with the CSFs of AD patients and controls diluted 1:1 with the extracellular medium for 5 h following or not a 1 h pre-incubation with DesAb-O at 3 μM. Unlike control CSFs, the AD ones caused a significant permeabilization of the cellular membrane relative to untreated cells, albeit to a lesser extent than ADDLs used as a positive control (Figure 3.9 C). Interestingly, DesAb-O can significantly prevent the membrane permeabilization induced by AD CSFs causing

an increase of intracellular calcein-derived fluorescence (by 23 ± 9%), although to a minor extent with respect to that evoked by ADDLs (by 41 ± 11%) (Figure 3.9 C). We also carried out an MTT reduction test following the administration of CSFs diluted 1:1 with the extracellular medium for 24 h. Unlike the control CSFs, those derived from AD patients caused a modest reduction of the mitochondrial activity of cells (by 19 ± 1%) that was reduced by the 1 h pre-incubation with 3 µM DesAb-O, evident as an improvement of mitochondrial function of 11 ± 2% (Figure 3.9 D). ADDLs caused a significant reduction of cell viability (by 36 ± 4%), that was prevented by DesAb-O, evident as an improvement of mitochondrial function of 15 ± 7% (Figure 3.9 D), confirming previous results shown in Figure 3.5 E.

Overall, these results confirmed the selective ability of DesAb-O to bind and neutralise both *in vitro* synthesised and patient-derived $A\beta_{42}$ oligomers, representing a promising tool for a future diagnostic, therapeutic and prognostic application in AD.

7 Chapter 3: figures and tables

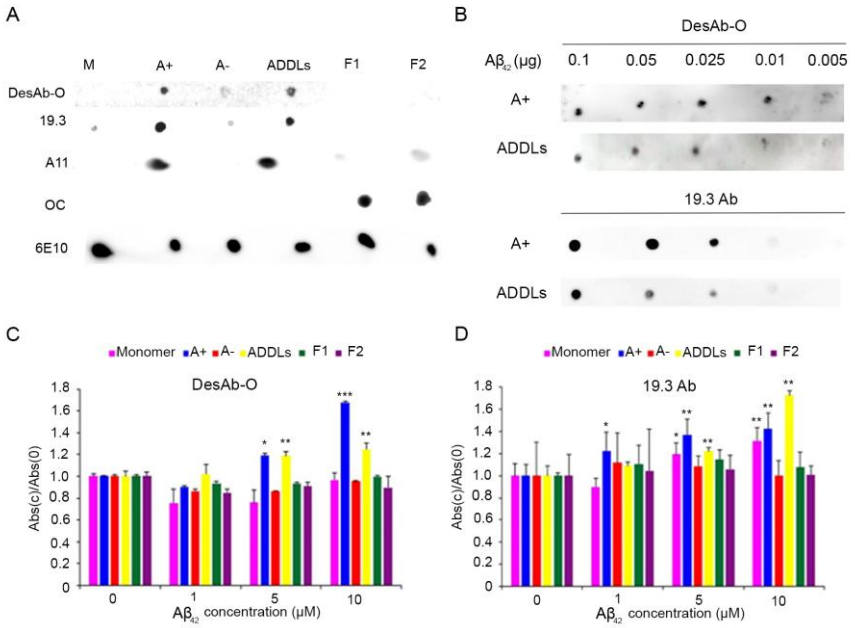

Figure 3.1 - DesAb-O selectively detects synthetic $A\beta_{42}$ oligomers *in vitro*. A-B) Dot-blot analysis of Aβ42 species. (A) Samples of monomeric $A\beta_{42}$ (M), oligomeric (A+,A- and ADDLs) and two type of fibrillar species (F1 from Ladiwala *et al.*, 2012 and F2 from Dahlgren *et al*., 2002) were deposited (2 µl/spot, corresponding to 0.1 µg) onto a nitrocellulose membrane and detected with the indicated Abs. (B) Samples of A+ oligomers and ADDLs at various amounts (0.25, 0.10, 0.05, 0.025, 0.01, 0.005 µg) were probed with DesAb-O (top) and 19.3 (bottom) Abs. C-D) ELISA measurements taken at increasing

concentration of $A\beta_{42}$ species using DesAb-O (C) and 19.3 (D) Abs. Data were normalized for the corresponding average value at concentration 0 μM. Experimental errors are S.D. (n = 3). Samples were analysed by Student t test relative to 0 μM (*P<0.05, ** P<0.01 and ***P<0.001).

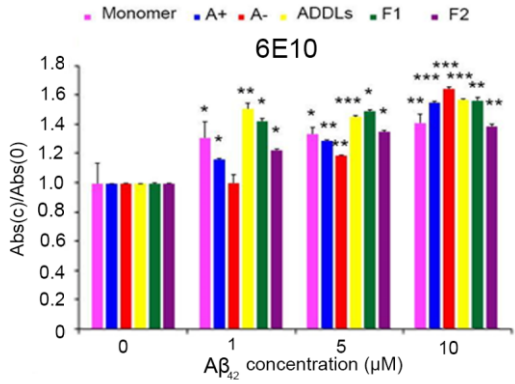

Figure 3.2 - 6E10 Ab detects all $A\beta_{42}$ species in a dose-dependent manner. Indirect ELISA measurements taken at increasing concentration of $A\beta_{42}$ species using the 6E10 Ab. Data were normalised for the corresponding average value at concentration 0 μM. Experimental errors are S.D. (n = 3). Samples were analysed by Student *t* test relative to 0 μM (*P<0.05, ** P<0.01 and ***P<0.001).

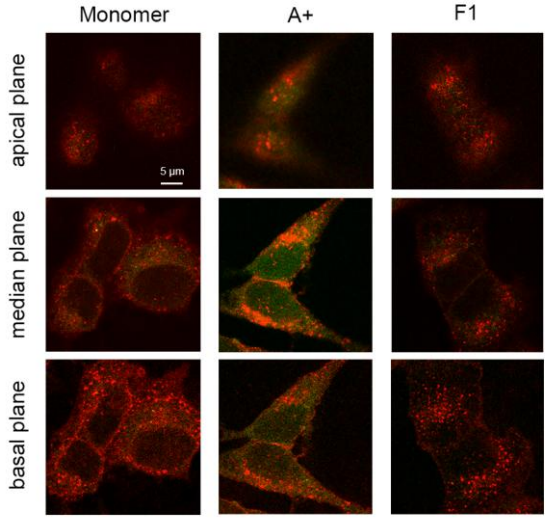

Figure 3.3 - DesAb-O detects $A\beta_{42}$ oligomers bound to the neuronal membrane and internalized into the cytosol. Representative STED microscopy images showing the basal, median, and apical sections of SH-SY5Y cells treated for 1 h with the indicated $A\beta_{42}$

species at 3.0 μM (monomer equivalents). Red and green fluorescence indicates respectively the cell membranes and the $A\beta_{42}$ species, detected with wheat germ agglutinin (WGA) and DesAb-O Ab.

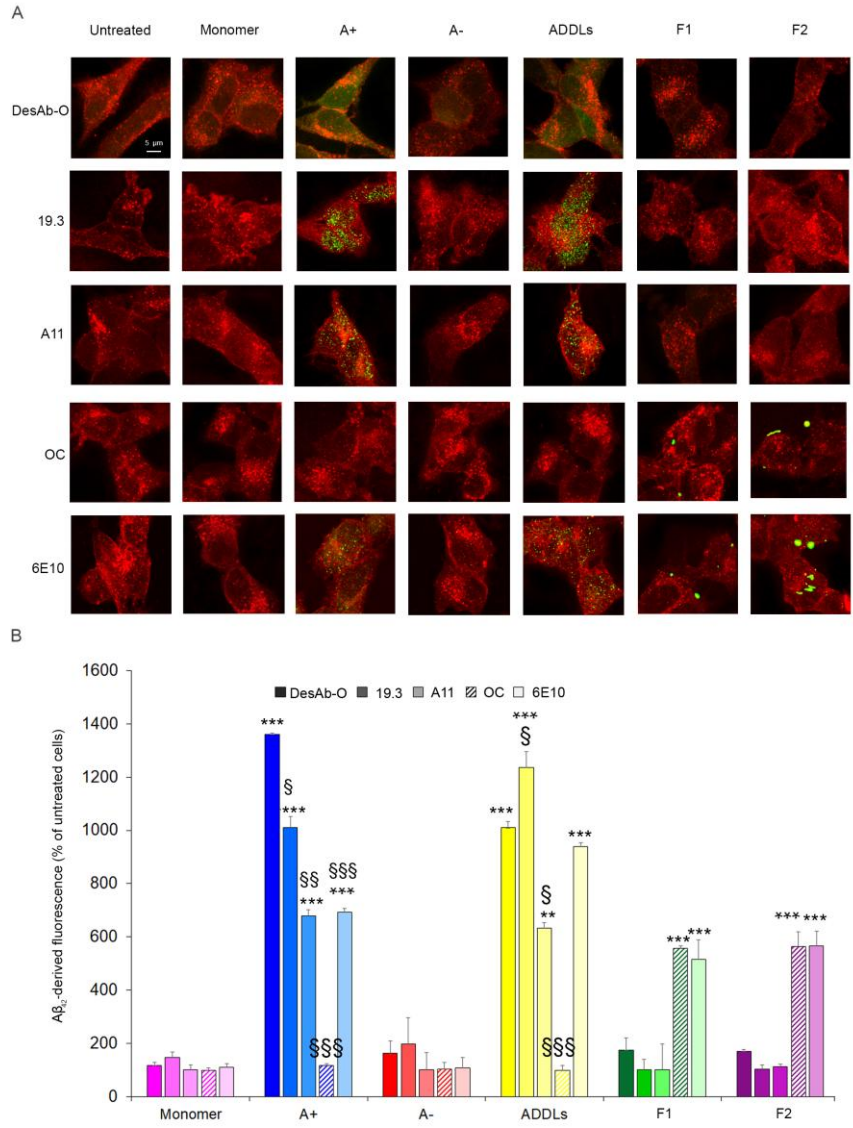

Figure 3.4 - DesAb-O detects synthetic $A\beta_{42}$ oligomers interacting with neuronal cells. A) Representative STED microscopy images of SH-SY5Y cells treated with the indicated Aβ42 species at 3.0 μM (monomer equivalents) for 1 h. Red and green fluorescence indicates respectively the cell membranes and the $A\beta_{42}$ species, detected with the indicated Abs. B) The results of a semi-quantitative analysis of the green fluorescent signal.

Experimental errors are S.E.M. (n = 3). Samples were analysed by Student *t* test relative to untreated cells (**P<0.01, and ***P<0.001), or to cells treated with the same $A\beta_{42}$ species and detected with DesAb-O (§P<0.05, §§P<0.01, §§§P P<0.001).

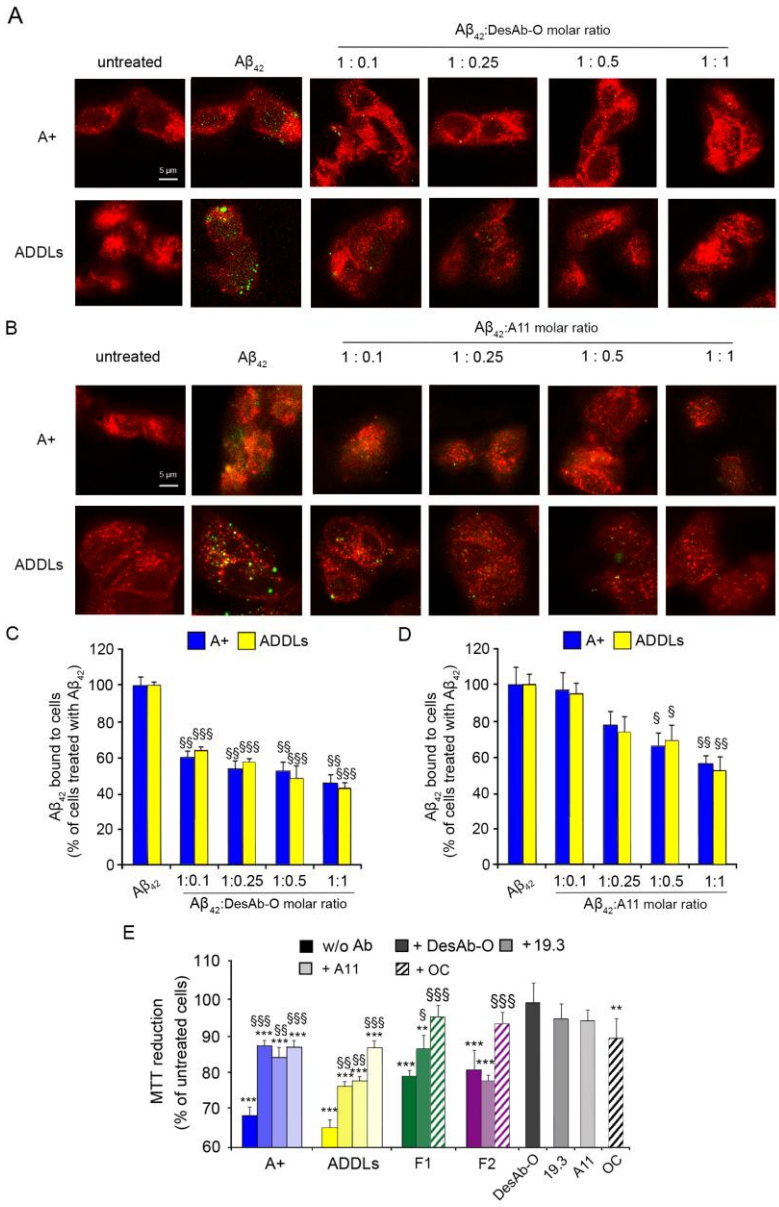

Figure 3.5 - DesAb-O inhibits the binding of $A\beta_{42}$ oligomers to the neuronal membrane

preventing their induced mitochondrial dysfunction. A-B). Representative confocal microscopy images of SH- SY5Y cells treated with 3.0 μM (monomer equivalents) A+ oligomers and ADDLs following 1 h pre- incubation in the absence or presence of DesAb-O (A) or A11 (B) Abs at the indicated $A\beta_{42}$:Abs molar ratios, where molar ratios refer to monomer equivalents. Red and green fluorescence indicates the cell membranes and $A\beta_{42}$ oligomers detected with the 6E10 Ab. C-D). Degree of membrane binding of A+ oligomers and ADDLs measured following incubation under the conditions represented in panels A and B, determined by using the ImageJ (NIH, Bethesda, MD, USA) and JACOP plugin (rsb.info.nih.gov) software. C) MTT reduction in SH-SY5Y cells treated for 24 h with the indicated $A\beta_{42}$ aggregates at a concentration of 3.0 μM (monomer equivalents) following a 1 h pre-incubation in the absence or presence of the indicated Abs. Abs alone were also tested as a control. Experimental errors are S.E.M. (n = 4). Samples were analysed by one-way ANOVA followed by Bonferroni's multiple-comparison test relative to untreated cells (*P<0.05, **P<0.01, and ***P<0.001), or to cells treated with the same $A\beta_{42}$ species without any Ab (§P<0.05, §§P<0.01, §§§P P<0.001). 200-250 (A-D) and 250,000-300,000 (E) cells were analysed per condition.

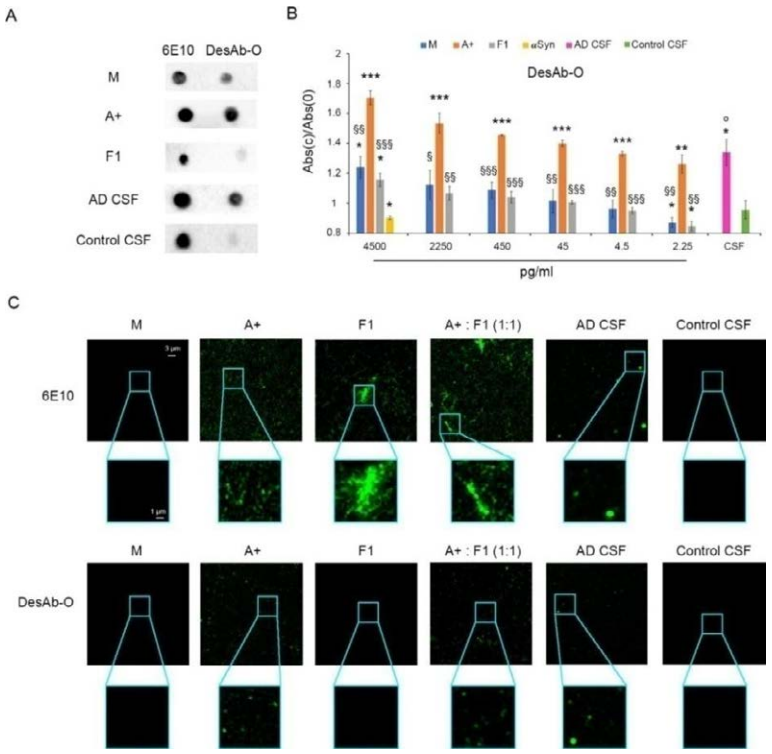

Figure 3.6 - DesAb-O detects $A\beta_{42}$ oligomers in the CSFs of AD patients *in vitro*. A) Representative sandwich dot-blot analysis of $A\beta_{42}$ species and CSFs. The capture Abs, 6E10 and DesAb-O, were spotted onto nitrocellulose membranes (2 μl corresponding to 1:100 and 10 μM). The membranes were incubated with solutions containing different $A\beta_{42}$ species (monomeric $A\beta_{42}$ (M), A+ oligomers, fibrils (F1) at 0.01 mg/ml, the CSF

from a representative AD patient (n = 9) and that from a representative control subject (n = 4) at 0.1 mg/ml. Finally, the membranes were probed with the detection 6E10 Ab. B) Sandwich indirect ELISA. 0.25 mg/ml of CSFs from AD patients and control subjects were adsorbed and quantified by using DesAb-O at 1 µM. Standard curve was obtained with decreasing concentration of $A\beta_{42}$ species formed *in vitro*. α-Synuclein monomeric protein was used as a negative control. Data were normalised for the corresponding average value at concentration 0 pg/ml. Experimental errors are S.E.M (n = 4 for synthetic samples and control CSFs and n = 9 for AD CSFs). Samples were analysed by Student *t* test relative to 0 pg/ml (* P<0.05, **P<0.01, *** P<0.001) or to A+ (§ P<0.05, §§ P<0.01, §§§P<0.001) or to control CSF (° P<0.05).C) Representative STED images showing $A\beta_{42}$ species (M, A+, F1, a mixture containing A+ and F1 at 1:1 molar ratio) and CSFs collected from AD patients and controls spotted in a glass coverslip at 25 µM and 0.5 mg/ml, respectively (n = 4 for synthetic samples and control CSFs and n = 9 for AD CSFs). The green fluorescent signals arise from the staining with 1:800 6E10 and 2 µM DesAb-O Abs. Higher magnifications of the $A\beta_{42}$ species are shown in the boxed areas.

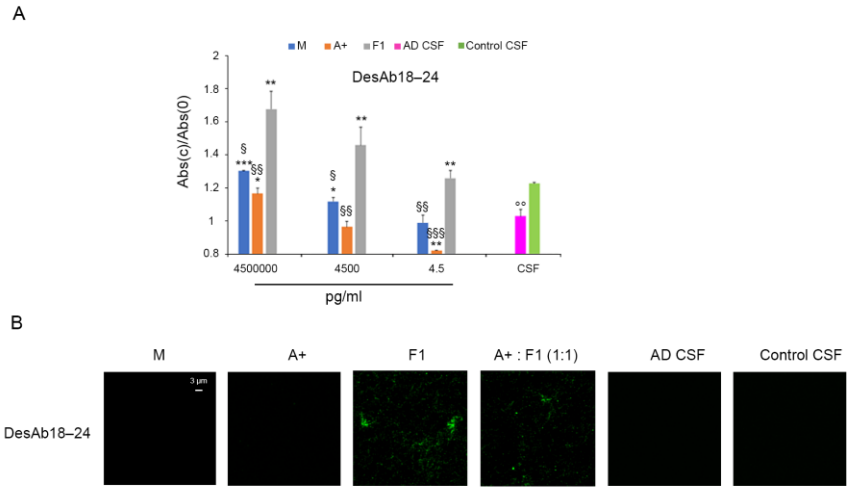

Figure 3.7 - $DesAb_{18–24}$ detects $A\beta_{42}$ fibrils and shows a non-specific signal in the CSF samples. A) Sandwich ELISA assay. 0.25 mg/ml of CSF samples from AD patients and control subjects were adsorbed and quantified using $DesAb_{18–24}$ at 0.5 µM. Standard curve was obtained with decreasing concentration of $A\beta_{42}$ species formed *in vitro*. Data were normalized for the corresponding average value at concentration 0 pg/ml). Experimental errors are S.E.M. (n = 4). Samples were analysed by Student *t* test relative to 0 pg/ml (* P<0.05, **P<0.01, *** P<0.001) or to F1 (§ P<0.05, §§ P<0.01, §§§P<0.001) or to control CSF (°° P<0.01). B) Representative STED images showing $A\beta_{42}$ species (M, A+ oligomers, F1, and a mixture containing both A+ and F1 at 1:1 molar ratio) and CSF samples collected from AD patients and controls (n = 4) spotted in a glass coverslip at 25 µM and 0.5 mg/ml, respectively. The green fluorescent signals arise from the staining with 4 µM $DesAb_{18–24}$.

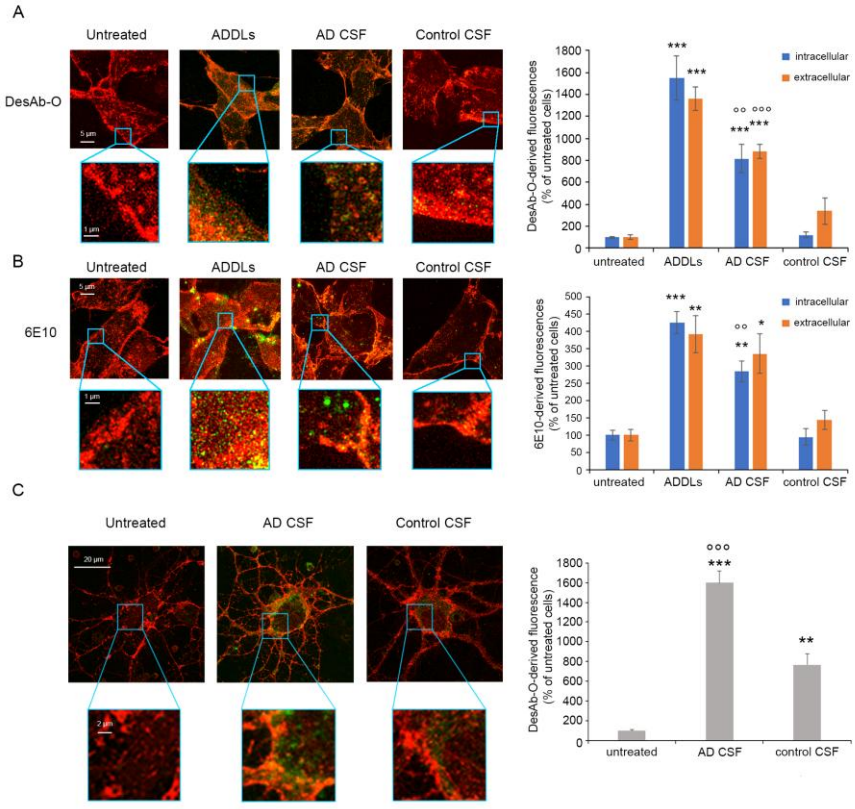

Figure 3.8 - DesAb-O detects $A\beta_{42}$ oligomers present in CSFs of AD patients upon their interaction with neuronal cells. A-B) Representative STED microscopy images of SH-SY5Y cells treated with ADDLs at 3.0 μM (monomer equivalents) or CSFs from AD patients and age-matched controls diluted 1:1 with the extracellular medium, for 5 h. Red and green fluorescence indicates the cell membranes and the $A\beta_{42}$ species detected with DesAb-O (A) and 6E10 (B), respectively. Higher magnifications of the $A\beta_{42}$ species are shown in the boxed areas. The histograms represent the results of a semi-quantitative analysis of the green fluorescent signal. C) Representative STED microscopy images of primary rat cortical neurons treated with AD and control CSFs, as reported in A-B. Red and green fluorescence indicates the cell membranes and the $A\beta_{42}$ species detected with DesAb-O, respectively. Higher magnifications of the $A\beta_{42}$ species are shown in the boxed areas. Experimental errors are S.E.M. (n = 4 for synthetic samples and control CSFs and n = 9 for AD CSFs). 200-250 cells were analysed per condition. Samples were analysed by Student *t* test relative to untreated cells (**P<0.01 and ***P<0.001), or to cells treated with control CSFs (°°P<0.01 and °°°P<0.001).

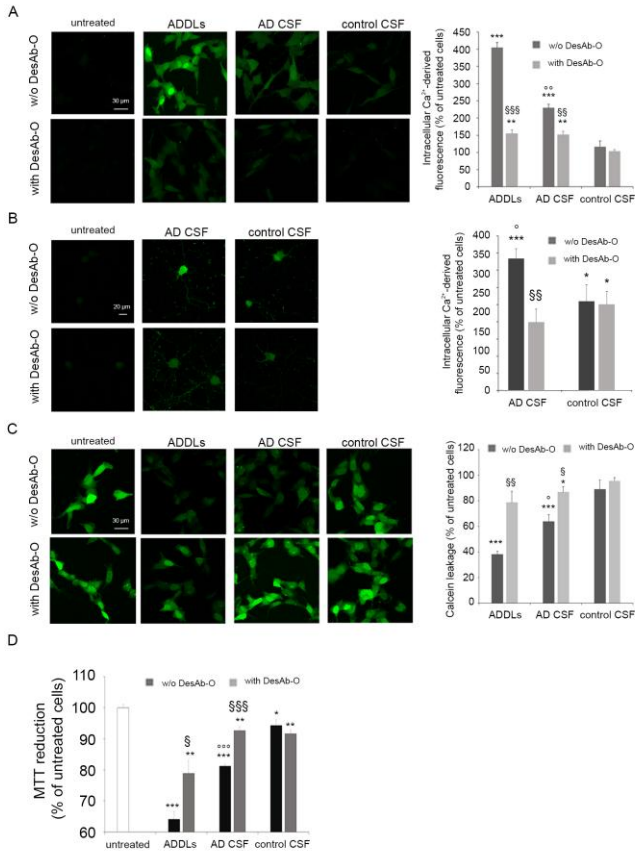

Figure 3.9 - DesAb-O prevents neuronal dysfunction induced by the CSFs of AD patients. A) Intracellular Ca^{2+}-derived fluorescence in SH-SY5Y untreated cells or treated for 5 h with ADDLs at 1 µM (monomer equivalents), or with CSFs from AD patients and age-matched control subjects (n = 4) following or not a 1 h pre-incubation in the absence or presence of DesAb-O at 3 µM. B) Intracellular Ca^{2+}-derived fluorescence in primary rat cortical neurons untreated or treated for 2.5 h with CSFs from AD patients and control subjects (n = 4) following or not a 1 h pre-incubation in the absence or presence of DesAb-O at 3 µM. C) Intracellular calcein-derived fluorescence in SH-SY5Y cells untreated or treated for 5 h with ADDLs at 1 µM (monomer equivalents), or with CSFs from AD patients and control subjects (n = 4) following or not a 1 h pre-incubation in the absence or presence of DesAb-O at 3 µM. D) MTT reduction in SH-SY5Y cells treated for 24 h with ADDLs at 1 µM (monomer equivalents), or with CSFs from AD patients and control subjects (n = 4) following or not a 1 h pre-incubation with DesAb-O at 3 µM. Experimental errors are S.E.M. Samples were analysed by Student *t* test relative to untreated cells (* P<0.05, ** P<0.01 and ***P<0.001) or to cells treated with samples without DesAb-O (§P<0.05, §§P<0.01 and §§§P<0.001) or to cells treated with control CSFs (°P<0.05, °°P<0.01 and °°°P<0.001). 200-250 (A,C),80-150 (B) and 250.000-300.000 (D) cells were analysed per condition.